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(54) Titre : BRASSICA JUNCEA PRESENTANT UNE TOLERANCE AUX HERBICIDES ET METHODE DE PRODUCTION

(54) Title: HERBICIDE TOLERANT BRASSICA JUNCEA AND METHOD OF PRODUCTION

(57) Abrégé/Abstract:
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STABLE HERBICIDE TOLERANT BRASSICA JUNCEA

Abstract of the Disclosur

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Title: Herbicide Tolerant Brassica Juncea and Method of Production

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FIELD OF THE INVENTION

The invention is in the field of Brassica juncea breeding (i.e., Brassica), specifically relating to the development of stable herbicide tolerant Brassica juncea lines, plants and plant parts. A method of producing stable herbicide tolerant Brassica juncea lines, plants and plant parts is also provided.

BACKGROUND OF THE INVENTION

Several Brassica species are recognized as an increasingly important oilseed crop and a source of high quality protein meal in many parts of the world. The oil extracted from the seeds commonly contains a lesser concentration of endogenously formed saturated fatty acids than other vegetable oils and is well suited for use in the production of salad oil or other food products or in cooking or frying applications. The oil also finds utility in industrial applications. Additionally, the meal component of the seeds can be used as a nutritious protein concentrate for livestock.

The three primary Brassica species currently utilized for Brassica production and development are Brassica napus, Brassica rapa and Brassica juncea, each of which belong to the family Brassicaceae. Brassica juncea is currently grown as an oilseed in India and China. As Brassica juncea tolerates heat and drought conditions to a greater extent than Brassica napus and Brassica rapa, there is potential for Brassica juncea production in certain areas of the United States, Canada and Australia. Table 1 contains a comparative description of the general characteristics of Brassica napus, Brassica rapa and Brassica juncea compiling information from the Canola Council of Canada worldwide web site and the USDA circular number C857 by Albina Musil USDA1950C857 (1951).

Brassica juncea is commonly grown as a condiment mustard species in several countries including Canada, Hungary, Poland, Ukraine, China, Nepal and India. Mustard quality Brassica juncea is typically high in glucosinolate and high in erucic acid content, but is relatively low in oil content. Mustard seed can be used in whole seed or crushed form. Seed may be milled into flour or the oil may be extracted for use in cooking. High glucosinolate and high erucic acid types are quality variants within the same species,

differing only in quality param ters. As a result, cross breeding between low and high glucosinolate or rucic acid genotypes are easily mad .

Certain genotypes of Brassica juncea generally possess relatively low erucic acid levels in the oil and low glucosinolate levels in the meal. Therefore, certain commercial varieties of Brassica juncea may be developed that can be termed "CANOLA®" in accordance with the ***trademark of the Canola Council of Canada, which refers to forms of oilseed Brassica with erucic acid of <2% in the oil and total glucosinolates of <30 micromoles/gram of defatted meal.***

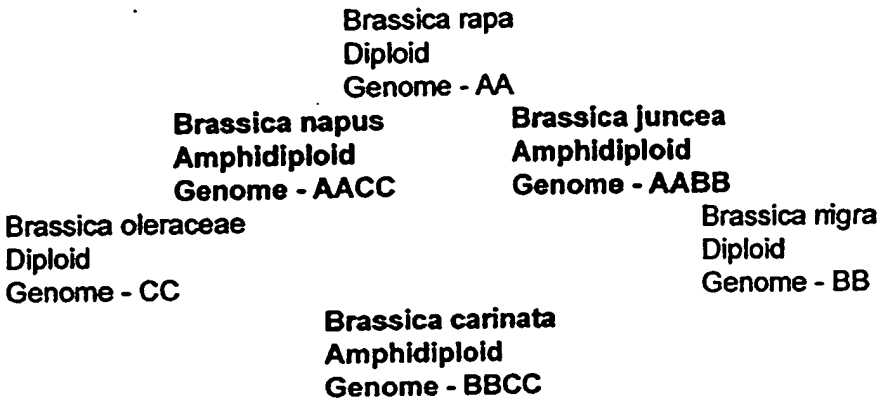
Table 1: Key morphological differences separating Brassica napus, Brassica juncea and Brassica rapa oilseeds and mustards

Trait/ Species	Brassica napus	Brassica juncea	Brassica rapa
Growth habit	• Spring and Winter	• Spring	• Spring and Winter
Cotyledon morphology	• Smooth on underside • Large – 5/8 to 7/8 inches across • Heart-shaped cotyledon and dark green in color	• Small – 5/16 to 9/16 inch across • Less lobed than napus – lighter green color	• Spiny and wrinkled on underside • Small – 5/16 to 9/16 inch across • Less lobed than napus – lighter green color
First leaf morphology	• Oblong or shield shaped, thin, bluish-green in color, smooth with a few hairs near the margin	• Oblong, bright green and hairy	• Oblong, bright green to light bluish-green, sparingly hairy
Flowers	• Buds borne above open flowers	• Open flowers borne above buds	• Compact bud clusters, buds held below uppermost open flowers
Pollination	• Principally self-pollinating and mostly self-compatible	• Principally self-pollinating and mostly self-compatible	• Principally cross- pollinated and self-incompatible (although there is one self-pollinating, self-compatible variety known as Yellow sarson)
Leaf morphology	• Leaf blade only partially clasps stem • Lyrate in form	• Small petiole attaches leaf to stem • Margins with irregular shallow indentations	• Leaf blade clasps stem completely • Roughly oblong with coarsely toothed margins
Seed color	• Black	• Brown and / or yellow	• Brown and / or yellow
Ploidy	Amphidiploid (AACC) • 2 copies of rapa genome (AA) • 2 copies of oleraceae genome (CC)	Amphidiploid (AABB) • 2 copies of rapa genome (AA) • 2 copies of nigra genome (BB)	Diploid (AA) • 2 copies of rapa genome (AA)

The genomic composition of canola species are as follows (Figure 1). Brassica rapa, a diploid species, contains only the A (rapa) genome and has a genomic

constitution of AA. Brassica napus is an amphidiploid with th rapa (A) and oleraceae (C) genomes and is listed as AACC. Brassica juncea is also an amphidiploid with the rapa (A) genome and the nigra (B) genom . Genetically, Brassica juncea is listed as AABB.

Figure 1: Genomic constitution of certain Brassica species (U, 1935). Amphidiploids listed in bold text



During pollen and ovule formation, the chromosomes within each genome will pair with their homologues (i.e., 'A' chromosomes will pair with 'A', 'B' will pair with 'B'), and it is extremely rare to have pairing of A and B or A and C. This pairing may be forced by repeated crossing and careful selection of plant phenotype during breeding, although there is no expectation that a trait from one genome may be combined with a trait from the other genome.

Brassica sp. cultivars are developed through breeding programs that utilize techniques such as mass and recurrent selection, backcrossing, pedigree breeding and haploidy. Recurrent selection is used to improve populations of either self- or cross-pollinating Brassica. Through recurrent selection, a genetically variable population of heterozygous individuals is created by intercrossing several different parents. The best plants are selected based on individual superiority, outstanding progeny, or excellent combining ability. The selected plants are intercrossed to produce a new population in which further cycles of selection are continued. Various recurrent selection techniques are used to improve quantitatively inherited traits controlled by numerous genes.

Breeding programs use backcross breeding to transfer genes for a simply inherited, highly heritable trait into another line that serves as th recurrent parent. Th source of th trait to be transferred is called the donor parent. After the initial cross, individual plants possessing the desired trait of the donor parent are selected and are crossed (backcrossed) to the recurrent parent for several generations. The resulting plant is expected to have the attributes of the recurrent parent and the desirable trait transferred from the donor parent. This approach has been used for breeding disease resistant phenotypes of many plant species. However, certain traits are difficult to transfer by backcross breeding because other attributes of the recurrent parent are linked to the desirable trait, and therefore it is difficult to develop a resulting plant with all of the attributes of the recurrent parent and the desirable trait transferred from the donor parent. Backcrossing has been used to transfer low erucic acid and low glucosinolate content into lines and breeding populations of Brassica.

Pedigree breeding and recurrent selection breeding methods are used to develop lines from breeding populations. Pedigree breeding starts with the crossing of two genotypes, each of which may have one or more desirable characteristics that is lacking in the other or which complements the other. If the two original parents do not provide all of the desired characteristics, other sources can be included in the breeding population. In the pedigree method, superior plants are selfed and selected in successive generations. In the succeeding generations the heterozygous condition gives way to homogeneous lines as a result of self-pollination and selection. Typically in the pedigree method of breeding five or more generations of selfing and selection is practiced: F_1 to F_2; F_2 to F_3; F_3 to F_4; F_4 to F_5, etc. For example, two parents that are believed to possess favorable complementary traits are crossed to produce an F_1. An F_2 population is produced by selfing one or several F_1's or by intercrossing two F_1's (i.e., sib mating). Selection of the best individuals may begin in the F_2 population, and beginning in the F_3 the best individuals in the best families are selected. Replicated testing of families can begin in the F_4 generation to improve the effectiveness of selection for traits with low heritability. At an advanced stage of inbreeding (i.e., F_6 and F_7), the best lines or mixtures of phenotypically similar lines commonly are tested for potential release as new cultivars. Backcrossing may be used in conjunction with pedigree breeding; for example, a combination of backcrossing and pedigree breeding with recurrent selection has been used to incorporate blackleg resistance into certain cultivars of Brassica napus.

Plants that have been self-pollinated and selected for type for many generations become homozygous at almost all gene loci and produce a uniform population of true

breeding progeny. If desired, the haploidy method can also be used to extract homogeneous lines. A cross between two different homozygous lines produces a uniform population of hybrid plants that may be heterozygous for many gene loci. A cross of two plants each heterozygous at a number of gene loci will produce a population of hybrid plants that differ genetically and will not be uniform.

The choice of breeding or selection methods depends on the mode of plant reproduction, the heritability of the trait(s) being improved, and the type of cultivar used commercially (e.g., F_1 hybrid cultivar, pureline cultivar, etc.).

SUMMARY OF THE INVENTION

The invention is in the field of Brassica juncea (i.e. Brassica) breeding, specifically relating to the development of stable herbicide tolerant Brassica juncea lines, plants and plant parts. A method of producing stable herbicide tolerant Brassica juncea lines, plants and plant parts is also provided.

DEFINITIONS

In the description and tables which follow a number of terms are used. In order to aid in a clear and consistent understanding of the specification the following definitions and evaluation criteria are provided.

Cotyledon. A cotyledon is a type of seed leaf that is contained on a plant embryo. A cotyledon contains the food storage tissues of the seed. The embryo is a small plant contained within a mature seed.

Cotyledon Length. The distance between the indentation at the top of the cotyledon and the point where the width of the petiole is approximately 4 mm.

Cotyledon Width. The width at the widest point of the cotyledon when the plant is at the two to three-leaf stage of development (mean of 50).

Fatty Acid Content: The typical percentages by weight of fatty acids present in the endogenously formed oil of the mature whole dried seeds are determined. During such determination, the seeds are crushed and are extracted as fatty acid methyl esters following reaction with methanol and sodium methoxide. Next the resulting ester is analyzed for fatty acid content by gas liquid chromatography using a capillary column which allows separation on the basis of the degree of unsaturation and fatty acid chain

length. This procedure is described in the work of J.K. Daun t al. J. Amer. Oil Chem. Soc., 60: 1751 to 1754 (1983) which is herein incorporated by reference.

Flower Bud Location. A determination is mad whether typical buds are disposed above or below the most recently opened flowers.

Glucosinolate Content. The total aliphatic glucosinolate content of the meal of the seeds is determined on the moisture free air-dried-oil-free solid meal as measured by the gas liquid chromatography method of the Canadian Grain Commission as is expressed micromoles per gram. Capillary gas chromatography of the trimethylsityl derivatives of extracted and purified desulfoglucosinolates with optimization to obtain optimum indole glucosinolate detection as described in *"Procedures of the Western Canada Canola/Rapeseed Recommending Committee Incorporated for the Evaluation and Recommendation for Registration of Canola/Rapeseed Candidate Cultivars in Western Canada"*.

Growth Habit. This refers to whether the Brassica is primarily a spring annual or winter annual type.

Herbicide Tolerance. Tolerance to various herbicides when applied at standard recommended application rates is expressed on a scale of 1 (highly tolerant), 2 (tolerant), or 3 (susceptible).

Leaf Morphology. Includes characteristics such as leaf attachment to stem, leaf color, leaf dentation, leaf margin hairiness. Often observed on first leaves and again when at least 6 leaves of the plant are completely developed.

Mutagenesis. Any one of many techniques known in the art to create or induce genetic mutations, including, without limitation, microspore mutagenesis as described in Swanson et al., *Plant Cell Reports 7:83-87* (1989).

Oil Content. The typical percentage by weight oil present in the mature whole dried seeds is determined by ISO 10565:1993 Oilseeds Simultaneous determination of oil and water - Pulsed NMR method.

Plant Height. The overall plant height at the end of flowering is observed (mean of 50).

Ploidy. This refers to whether the number of "basic sets" of chromosomes (individual replicates of the same genome) exhibited by the cultivar is diploid (two sets) or amphidiploid (two sets each of two different genomes).

Resistance to Shattering. Resistance to silique shattering is observed at seed maturity and is expressed on a scale of 1 (poor) to 5 (excellent).

Seed Coat Color. The seed coat color of typical mature seeds is observed.

DETAILED DESCRIPTION OF THE INVENTION

A Brassica breeding population should be substantially homogenous and reproducible to be useful in either further breeding or the development of a commercial cultivar. There are a number of analytical methods available to determine the phenotypic stability of a Brassica population.

The oldest and most traditional method of analysis is the observation of phenotypic traits. The data is usually collected in field experiments over the life of the Brassica plants to be examined. Phenotypic characteristics most often are observed for traits associated with seed yield, seed oil content, seed protein content, fatty acid composition of oil, glucosinolate content of meal, growth habit, lodging resistance, plant height, shattering resistance, etc. Other phenotypic characteristics commonly observed include resistance to disease, insects and tolerance to herbicides. Herbicide tolerance is particularly important for Brassica, since many weeds, such as stinkweed, shepherd's purse, flixweed, ball mustard, wormseed mustard, hare's ear mustard and common peppergrass have a close genetic relationship with Brassica. Therefore, it is advantageous for a cultivar to have herbicide tolerance not possessed by related weeds or even undesired Brassica plants of a different variety or cultivar.

Herbicides may function by disrupting amino acid biosynthesis in affected species. For example, the imidazolinone herbicides are active on the enzyme acetohydroxy acid synthase (AHAS), the first enzyme in the biosynthesis of the amino acids leucine, isoleucine and valine. Imidazolinone herbicide tolerance prevents inhibition of the AHAS enzyme, allowing tolerant plants to continue with normal amino acid biosynthesis.

Most forms of Brassica napus, Brassica rapa and Brassica juncea are highly susceptible to herbicides, such as imidazolinones. Doses of imidazolinone herbicides applied during our backcross breeding program were sufficient to kill susceptible

Brassica juncea and Brassica napus checks as well as material segregating away from herbicide tolerance.

Swanson et al., *Plant Cell Reports 7:83-87* (1989) reported the development of imidazolinone herbicide tolerant Brassica napus mutants using microspore mutagenesis. During the process, five fertile double-haploid Brassica napus plants were developed. One of the mutants was tolerant to between 5 and 10 times recommended field rates of an imidazolinone herbicide. An inheritance study indicated that two semi-dominant unlinked genes combined to develop an F1 with greater tolerance than either of the parents. The mutants were subsequently crossed with other breeding material to develop Pioneer variety 46A72.

Rutledge et al. *Mol. Gen. Genet. 229:31-40* (1991) proposed a model for the inheritance of the AHAS genes in Brassica napus. AHAS2, AHAS3 and AHAS4 appear to be linked with the A (rapa) genome and AHAS1 and AHAS5 are likely associated with the C (oleraceae) genome. AHAS1 and AHAS3 were expressed at all growth stages (Ouellet et al., *Plant J. 2:321-330* 1992) and mutant forms of AHAS1 and AHAS3 appear to be the most effective tolerance genes. AHAS2 was found to be active only in ovules and seeds. AHAS4 was found to be defective due to interrupted sequences in the middle of the coding region (Rutledge et al., *Mol. Gen. Genet. 229:31-40*, 1991) and was not expressed in tissues examined by Ouellet et al. *Plant J. 2:321-330*, (1992). The last gene, AHAS5, may also be defective (Rutledge et al. *Mol. Gen. Genet. 229:31-40*, 1991). Hattori et al. *Can J. Bot: 70: 1957-1963,* (1992) determined that the DNA sequence of coding regions for AHAS1 and AHAS3 were 98% identical. DNA sequences of the 5' and 3' ends were also closely related. Few genetic similarities were observed between the sequences of AHAS2 as compared to AHAS1 or AHAS3 genes.

Thus, there are only two known effective genes for imidazolinone herbicide tolerance – an AHAS1 mutant (believed to be located on the C genome) and an AHAS3 mutant (believed to be located on the A genome). As Brassica juncea, Brassica napus and Brassica rapa all contain the A genome (Figure 1), transfer of the AHAS3 mutant gene is a simple matter of crossing the species and selecting under herbicide selection as normal genome recombinations would likely allow for the complete transfer of the mutant AHAS3 coding segments. A single AHAS tolerance gene will provide some protection under very low screening rates of herbicides. Under high screening rates (such as were used in the greenhouse and field screening protocols), genotypes possessing the single AHAS1 tolerance gene do not die, but are severely stunted, grow multiple racemes and are very late to flower and mature. In our normal screening

program, we discarded these individuals. Accordingly, both AHAS1 and AHAS3 mutant genes appear to be required for th levels of tolerance evaluated in these experiments.

Because mutant forms of both genes appear to be required for full tolerance, the mutant AHAS3 gene and the mutant AHAS1 gene (believed to be on the C genome) must be transferred into the same genotype. Brassica juncea and Brassica rapa do not contain the C genome, which is one reason why there have been no commercial herbicide tolerant Brassica juncea and Brassica rapa varieties developed to date. Herbicide tolerance provided by only a single mutant AHAS gene is insufficient to protect otherwise susceptible plants from field application rates of herbicides. Attempts up until this research to transfer the mutant AHAS1 gene and the corresponding herbicide tolerance trait to Brassica rapa and Brassica juncea have proven to be very difficult.

In the present invention, herbicide tolerant Brassica juncea was developed through a backcross breeding program. 46A72, a Pioneer Hi-Bred herbicide tolerant Brassica napus commercial variety, was used as the trait donor. The herbicide tolerance trait in 46A72 was developed through mutagenic techniques described by Swanson, et al. (1989). PHI research has confirmed that there is some level of cross-tolerance between the tolerance sources for imidazolinone herbicides and sulfonyl urea herbicides. 46A72 (the donor parent) has been shown to be tolerant to sulfonyl urea herbicides as well as imidazolinone herbicides. 46A72 is available from Pioneer Hi-Bred International, Inc., 400 Locust Street, Des Moines, Iowa 50309.

The first step in the development of the herbicide tolerant Brassica juncea lines was to cross the herbicide tolerant Brassica napus variety 46A72 with low glucosinolate and low erucic acid Brassica juncea lines (Figure 2) using the Brassica juncea parents as females. The seeds and plants resulting from this cross are referred to as the F1 generation. The F1 generation was used as a female to receive pollen from Brassica juncea lines to develop the BC1 generation. The BC1 was used as a female parent to receive pollen from Brassica juncea lines to produce the BC2. A third round of crossing was used to produce the BC3. The F1, BC1 and BC2 generations were screened for herbicide tolerance by using Pursuit® herbicide at a rate of 50ml / ha (1x field rate), or Odyssey® herbicide at a rate of 30g / ha (1x field rate). Both herbicides are available from American Home Products, Inc., American Cyanamid Division, 5 Giralda Farms, Madison, New Jersey, 07940, and Pursuit® and Odyssey® are trademarks owned by American Home Products, Inc. Plants exhibiting satisfactory levels of herbicide tolerance during the herbicide tolerance program were crossed and selected. The screening, crossing and selection was repeated, and the first stable herbicide tolerant Brassica

juncea phenotypes (designated 98SJ-23841, 98SJ-23844 and 98SJ-23845) were produced at the BC3 generation. Each of lines 98SJ-23841, 98SJ-23844 and 98SJ-23845 are substantially stable and reproducible for both herbicide tolerance and the Brassica juncea phenotype.

There were other related BC3 materials (unstable sister populations of 98SJ-23841, 98SJ-23844 and 98SJ-23845) that varied for herbicide tolerance and plant phenotype. These BC3 materials exhibited less stability for the Brassica juncea phenotype as they ranged from plants with a complete Brassica napus phenotype to plants with a complete Brassica juncea phenotype. There were also a large number of plants in the sister populations that exhibited various combinations of plant traits from either Brassica napus or Brassica juncea. These unstable sister populations continued to segregate for plant phenotype traits with continued selfing and evaluation. The unstable sister populations also segregated for herbicide tolerance as plants ranged between complete tolerance, intermediate tolerance and full susceptibility to the herbicide. During the evaluation of fully tolerant materials prior to the BC3 generation, we found no evidence of complete tolerance associated with a complete Brassica juncea phenotype. Many of the intermediate tolerance plants had Brassica juncea characteristics, but had insufficient levels of tolerance to protect the plants from herbicide damage during screening and evaluation.

The second step in developing the herbicide tolerance was to verify stability in subsequent generations (Figure 3).

Unexpected difficulties were encountered in the backcross breeding program as a result of the linkage between the Brassica napus phenotype and the herbicide tolerant trait. During the first three rounds of crossing (F1, BC1 and BC2) all of the plants which inherited the herbicide tolerance trait also inherited the Brassica napus phenotype, or their selfed progeny reverted back to the Brassica napus phenotype in subsequent generations. Conversely, plants that did not inherit the herbicide tolerance trait inherited the Brassica juncea phenotype. Thus, it was only through great effort involving many crosses and careful selection within BC2 segregating materials that the three populations (98SJ-23841, 98SJ-23844 and 98SJ-23845) were developed that expressed both the Brassica juncea phenotype and the herbicide tolerance trait. Presumably, the necessary genetic change that allowed for the simultaneous expression of a fully fertile Brassica juncea plant phenotype in combination with herbicide tolerance occurred between the BC2 and BC3.

98SJ-23841, 98SJ-23844 and 98SJ-23845 were the first populations to show uniformity and stability for the juncea phenotype and herbicid tolerance. Each of these three populations demonstrated a substantial degree of herbicide tolerance. Successive selfed progenies derived from these three backcross populations have also exhibited stable herbicide tolerance and continue to maintain the Brassica juncea phenotype under greenhouse and field evaluation. In addition, it is known to those skilled in the art that herbicide tolerance genes in Brassica napus commonly confer cross-tolerance to sulfonyl urea herbicides.

Figure 2: Breeding procedure used to develop herbicide tolerant Brassica juncea

Females	Male
Bulk population from 16 Brassica juncea breeding lines low glucosinolate (9-18 umoles) low erucic acid (<1%)	46A72
Crossed to produce the F1	
Female	Males
F1 from previous cross 13 F1 lines x 15 plants per line Selected with Pursuit® 50ml/ha a.i. Chose resistant plants for crossing	Bulk pollen from 16 breeding lines – F5 to F8 generation low glucosinolate (<8 um) low erucic acid (< 0.5%)
Crossed to produce BC1	
Female	Males
BC1 populations from previous cross 6 BC1 populations x 36 plants per line Selected with Pursuit® - 50 ml/ha a.i. Chose resistant plants for crossing	Bulk pollen from16 breeding lines – F5 to F8 generation low glucosinolate (<8 umoles) low erucic acid (<0.5%)
Crossed to produce BC2	
Female	Males
BC2 seed from previous cross 4 BC populations Selected with Pursuit® – 50 ml /ha a.i. Chose resistant plants for crossing	Bulk pollen from 3 breeding lines – F6 generation low glucosinolate (6 to 12 umoles) low erucic acid (<0.5%)
Crossed to produce BC3	
Stable juncea phenotype combined with Pursuit® tolerance Lines coded: 98SJ-23841, 98SJ-23844, 98SJ-23845	

Figure 3: Greenhous and fi ld evaluation f H rbicid tolerant Brassica juncea populations

Greenhouse evaluation 1 – verify tolerance and juncea phenotype

98SJ-23841, 98SJ-23844, 98SJ-23845 and unstable BC3 sister lines planted for herbicide tolerance evaluation

Pursuit® applied at 50 ml/ha a.i.; juncea phenotype stable

Survivors self pollinated and harvested

Greenhouse evaluation 2 – verify tolerance and juncea phenotype

Survivors from previous project planted for herbicide tolerance evaluation

Pursuit® applied at 50 ml/ha a.i.; juncea phenotype and tolerance stable in 98SJ-23841, 98SJ-23844 and 98SJ-23845

Survivors self pollinated and harvested

Field evaluation 1 – verify tolerance and juncea phenotype under field conditions

Pioneer Hi-Bred International Puerto Vallarta Mexico Research Station

Self-pollinated selections from all other previous projects were planted at a single location

Odyssey® was applied at 30g/ha a.i.

Juncea phenotype stable – tolerance present in 98SJ-23841, 98SJ-23844 and 98SJ-23845 progenies

Other material derived from other generations and breeding lines exhibited a range of tolerance ranging from fully resistant, intermediate resistant and susceptible. Plant phenotypes ranged from full Brassica napus to Brassica juncea phenotypes and lines and populations that exhibited traits that were intermediate between Brassica napus and Brassica juncea. In these other materials, full resistance to the herbicide was not associated with the juncea phenotype, and vice-versa.

As can be seen in Table 2, the stable herbicide resistant lines 98SJ-23841, 98SJ-23844 and 98SJ-23845 are phenotypically similar to Brassica juncea line 96SJ-3827 used in their development. They are also phenotypically different from Brassica napus trait donor 46A72, with the exception that they possess the herbicide tolerance trait of 46A72.

Table 2: Phenotypic descripti ns f 46A72 (Brassica napus d n r), a junc a breeding line used in th backcross procedure (96SJ-3827) and three breeding populations (98SJ-23841, 98SJ-23844 and 98SJ-23845) developed during the breeding process.

Trait	Brassica napus trait donor 46A72	98SJ-23841	98SJ-23844	98SJ-23845	96SJ-3827 – Brassica juncea parent in BC1 and BC2 stage
Growth habit	• Spring	• Spring	• Spring	• Spring	• Spring
Cotyledon morphology	• Large – 5/8 to 7/8 inches across • Heart-shaped cotyledon and dark green in color	• Small – 5/16 to 9/16 inch across • Light green color	• Small – 5/16 to 9/16 inch across • Light green color	• Small – 5/16 to 9/16 inch across • Light green color	• Small – 5/16 t 9/16 inch across • Light green color
First leaf morphology	• Bluish-green in color, smooth with a few hairs near the margin	• Bright green and hairy	• Bright green and hairy	• Bright green and hairy	• Bright green and hairy
Flowers	• Buds borne above open flowers	• Buds borne below open flowers	• Buds borne below open flowers	• Buds borne below open flowers	• Buds borne below open flowers
Pollination	• Self-pollinating and self-fertile	• Self-pollinating and self-fertile	• Self-pollinating and self-fertile	• Self-pollinating and self-fertile	• Self-pollinating and self-fertile
Leaf morphology	• Leaf blade only partially clasps stem • Blue-green in color	• Small petiole attaches leaf to stem • Bright green color	• Small petiole attaches leaf to stem • Bright green color	• Small petiole attaches leaf to stem • Bright green color	• Small petiole attaches leaf to stem • Bright green color
Pods	• Larger and fewer pods • Medium length beak	• Small pods - 14-16 seeds per pod • Long beak and flattened pods	• Small pods - 14-16 seeds per pod • Long beak and flattened pods	• Small pods - 14-16 seeds per pod • Long beak and flattened pods	• Small pods – 14-16 seeds per pod • Long beak and flattened pods
Resistance to shattering	• Easily shattered when ripe	• Resistant to shattering under greenhouse conditions	• Resistant to shattering under greenhouse conditions	• Resistant to shattering under greenhouse conditions	• Resistant to shattering under field and greenhouse conditions
Seed color	• Black	• Yellow	• Yellow Brown	• Brown Yellow	• Yellow

MORPHOLOGICAL

The herbicide tolerant Brassica juncea populations are each substantially resistant to herbicides and can be reproduced by planting seeds of such lines, growing the resulting Brassica plants under self-pollinating or sib-pollinating conditions with adequate isolation, and harvesting the resulting seed using conventional agronomic practices.

DEVELOPMENT OF CULTIVARS

The stable herbicide tolerant Brassica juncea populations can be used to develop new herbicide tolerant Brassica juncea cultivars by any manner known to those skilled in the art, such as crossing with other Brassica juncea lines, followed by selfing and selection of plants with the desired characteristics. The stable herbicide tolerant Brassica juncea lines may also be used as either donor lines or recurrent parents as part of a breeding program. Exposure to herbicide can be used to determine inheritance of the herbicide tolerance trait. Similarly, where a marker exists for a known gene or gene product, the breeder may use the marker to assist in determining which germplasm has inherited the trait and is suitable for advancement to the next generation.

TRANSFORMATION OF BRASSICA

With the advent of recombinant DNA techniques that have allowed the isolation and characterization of genes that encode specific protein products, scientists in the field of plant biology developed a strong interest in engineering the genome of plants to contain and express foreign genes, or additional, or modified versions of native, or endogenous, genes (perhaps driven by different promoters) in order to alter the traits of a plant in a specific manner. Such foreign, additional and/or modified genes are referred to herein collectively as "transgenes", and plants containing one or more transgenes inserted into the plant genome through the use of recombinant DNA techniques are referred to as "transgenic plants." Over the last 15 to 20 years, several methods for producing transgenic plants have been developed, and the present invention, in particular embodiments, also relates to transformed versions of the claimed plant or line and to transformed versions of cultivars developed from such plant or line.

Plant transformation involves the construction of an expression vector, which will function in plant cells. Such a vector comprises a gene under control of or operatively linked to a regulatory element (for example, a promoter). The expression vector may contain one or more such operably linked gene/regulatory element combinations. The vector(s) may be in the form of a plasmid, and can be used alone or in combination with other plasmids, to provide transformed Brassica plants, using transformation methods known in the art to incorporate transgenes into the genetic material of the Brassica plant(s). Genes included in expression vectors must be driven by a nucleotide sequence comprising a regulatory element, for example, a promoter. Several types of promoters are now well known in the transformation arts, as are other regulatory elements that can be used alone or in combination with promoters. The term "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. Examples of promoters include promoters that preferentially initiate transcription in certain tissues,

such as leaves, roots, seeds, fibers, xylem vessels, tracheids, or sclerenchyma. A "constitutive" promoter is a promoter which is activ under most environmental conditions and in most tissue. With transgenic plants according to the present invention, a foreign protein can be produced in commercial quantities. Thus, techniques for the selection and propagation of transformed plants, which are well understood in the art, yield a plurality of transgenic plants which are harvested in a conventional manner, and a foreign protein then can be extracted from a tissue of interest or from total biomass.

Likewise, by means of the present invention, agronomic genes can be expressed in transformed plants. More particularly, plants can be genetically engineered to express various phenotypes of agronomic interest. Exemplary genes implicated in this regard include, but are not limited to, those categorized below.

1. Genes That Confer Resistance to Pests or Disease and that Encode
    (a) Plant disease resistance genes. Plant defenses are often activated by specific interaction between the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence (Avr) gene in the pathogen. A plant variety can be transformed with cloned resistance to engineer plants that are resistant to specific pathogen strains.
    (b) A gene conferring resistance to fungal pathogens, such a oxalate oxidase or oxalate decarboxylase.
    (c) A *Bacillus thuringiensis* protein, a derivative thereof or a synthetic polypeptide modeled thereon.
    (d) A lectin.
    (e) A vitamin-binding protein such as avidin.
    (f) An enzyme inhibitor, for example, a protease or proteinase inhibitor or an amylase inhibitor.
    (g) An insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof.
    (h) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest.
    (i) An insect-specific venom produced in nature by a snake, a wasp, etc.
    (j) An enzyme responsible for an hyperaccumulation of a monterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.

(k) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic nzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic.

(l) A molecule that stimulates signal transduction.

(m) A hydrophobic moment peptide.

(n) A membrane permease, a channel former or a channel blocker.

(o) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa, mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus.

(p) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect.

(q) A virus-specific antibody.

(r) A developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, funal endo -1, 4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo-α-1, 4-D-galacturonase.

(s) A developmental-arrestive protein produced in nature by a plant. For example, transgenic plants expressing the barley ribosome-inactivating gene have an increased resistance to fungal disease.

(t) Genes involved in the Systemic Acquired Resistance (SAR) Response and/or the pathogenesis related genes.

(u) Antifungal genes.

2. Genes That Confer Tolerance To A Herbicide

(a) A herbicide that inhibits the growing point or meristem, such as an imidazolinone or a sulfonyl urea. Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee et al., EMBO J. 7: 1241 (1988), and Miki et al., Theor. Appl.Genet. 80: 449 (1990), respectively. Lines 98SJ-23841, 98SJ-23844 and 98SJ-23845 are not

transgenic, although copies of mutant ALS or AHAS genes could be added to these lines by transgenic methods to further enhance their herbicide tolerance.

(b) Glyphosate (resistance imparted by mutant 5-enolpyruvl-3-ph sphikimate synthase (EPSP) and aroA genes, respectively) and other phosphono compounds such as glufosinate (phosphinothricin acetyl transferase, PAT) and Streptomyces hygroscopicus phosphinothricin-acetyl transferase, bar, genes), and pyridinoxy or phenoxy propionic acids and cycloshexones (ACCase inhibitor-encoding genes). See, for example, U.S. patent No. 4,940,835 to Shah et al., which discloses the nucleotide sequence of a form of EPSP which can confer glyphosate resistance. A DNA molecule encoding a mutant aroA gene can be obtained under ATCC accession No. 39256, and the nucleotide sequence of the mutant gene is disclosed in U.S. patent No. 4,769,061 to Comai. European patent application No. 0 333 033 to Kumada et al. and U.S. patent No. 4,975,374 to Goodman et al. disclose nucleotide sequences of glutamine synthetase genes which confer tolerance to herbicides such as L-phosphinothricin. The nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in European application No. 0 242 246 to Leemans *et al.* De Greef *et al.*, *Bio/Technology* 7: 61 (1989), describe the production of transgenic plants that express chimeric *bar* genes coding for phosphinothricin acetyl transferase activity. Exemplary of genes conferring tolerance to phenoxy propionic acids and cycloshexones, such as sethoxydim and haloxyfop, are the *Acc1-S1*, *Acc1-S2* and *Acc1-S3* genes described by Marshall *et al.*, *Theor. Appl. Genet.* 83: 435 (1992).

(c) A herbicide that inhibits photosynthesis, such as a triazine (*psbA* and *gs+* genes) and a benzonitrile (nitrilase gene). Przibilla *et al.*, *Plant Cell* 3: 169 (1991), describe the transformation of *Chlamydomonas* with plasmids encoding mutant *psbA* genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. patent No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC Accession Nos. 53435, 67441 and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes *et al.*, *Biochem. J.* 285: 173 (1992).

3. Genes That Confer or Contribute To a Grain Trait

(a) Modified fatty acid metabolism, for example, by transforming a plant with an antisense gene or stearoyl-ACP desaturase to increase stearic acid content of the plant.

(b) Decreased phytate content.

(c) Introduction of a phytase-encoding gene would enhance breakdown of phytate, adding more free phosphate to th transformed plant.

(d) A gen could be introduced that reduces phytate content. In maize, this, for example, could be accomplished by cloning and then reintroducing DNA associated with the single allele which is responsible for maize mutants characterized by low levels of phytic acid.

(e) Modified carbohydrate composition effected, for example, by transforming plants with a gene coding for an enzyme that alters the branching pattern of starch.

(f) Reduced green seed, by down regulation of the CAB gene in Brassica seed.

In addition to the categories noted above, genes that control pollination or self-compatibility may also be expressed in transformed plants.

Numerous methods for plant transformation have been developed, including biological and physical, plant transformation protocols. In addition, expression vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are available. These include, but are not limited to *Agrobacterium*-mediated transformation and direct gene transfer such as microprojectile bombardment or sonication. A transgenic variety produced by these methods could then be crossed, with another (non-transformed or transformed) variety, in order to produce a new transgenic variety. Alternatively, a genetic trait which has been engineered into a particular Brassica line using the foregoing transformation techniques could be moved into another line using traditional backcrossing techniques that are well known in the plant breeding arts. For example, a backcrossing approach could be used to move an engineered trait from a public, non-elite variety into an elite variety, or from a variety containing a foreign gene in its genome into a variety or varieties which do not contain that gene. As used herein, "crossing" can refer to a simple X by Y cross, or the process of backcrossing, depending on the context. It is also known in the art to culture Brassica cells or protoplasts, and to regenerate plants therefrom.

This invention also is directed to methods for producing a Brassica plant by crossing a first parent Brassica plant with a second parent Brassica plant wherein the first or second parent Brassica plant is one of the stable herbicide tolerant lines. Further, both first and second parent Brassica plants can be the same or different line. Thus, any such methods using the lines as a parent are within the scope of the present invention. Advantageously, the lines of the present invention can be used in crosses with other, different, Brassica inbreds to produce first generation (F_1) Brassica hybrid seeds and plants with superior characteristics.

As used herein, the term "plant" includes plant cells, plant protoplasts, plant cell tissue cultures from which Brassica plants can be regenerated, such as plant calli, plant clumps, and plant cells that are intact in plants or parts of plants, including embryos, pollen, ovules, flowers, pods, leaves, roots, root tips, anthers, stalks, and the like.

INDUSTRIAL APPLICABILITY

The seed of 98SJ-23841, 98SJ-23844, and 98SJ-23845, the plant produced from such seed, a hybrid Brassica plant produced from the crossing of any of these lines, the resulting hybrid seed, and various parts of the hybrid Brassica plant can be utilized in the production of an edible vegetable oil or other food products in accordance with known techniques. The remaining solid meal component derived from seeds can be used as a nutritious livestock feed. Brassica populations 98SJ-23841, 98SJ-23844 and 98SJ-23845 can also be used as breeding lines to develop herbicide tolerant Brassica (including canola and mustard quality) cultivars.

DEPOSITS

A deposit of the seed of 98SJ-23841, 98SJ-23844 and 98SJ-23845 is and has been maintained by Pioneer Hi-Bred International, Inc., 800 Capital Square, 400 Locust Street, Des Moines, Iowa 50309-2340, since prior to the filing date of this application. Access to this deposit will be available during the pendency of the application to the Commissioner of Patents and Trademarks and persons determined by the Commissioner to be entitled thereto upon request. Upon the maturation of this application into a patent, Applicant(s) will make available to the public without restriction a deposit of at least 2,500 seeds of each of 98SJ-23841, 98SJ-23844 and 98SJ-23845 deposited at the American Type Culture Collection (ATCC), Manassas, Virginia 20852. The seeds deposited with the ATCC will be taken from the same deposit maintained at Pioneer Hi-Bred International, Inc. and described above. Additionally, Applicant(s) will comply with all of the requirements of 37 C.F.R. §§1.801 - 1.809, including providing an indication of the viability of the sample when the deposit is made. This deposit of the 98SJ-23841, 98SJ-23844 and 98SJ-23845 populations will be maintained in the ATCC, which is a public depository recognized by the Budapest Treaty, for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent, whichever is longer, and will be replaced if it ever becomes nonviable during that period. More specifically, seeds of populations 98SJ-23841, 98SJ-23844 and 98SJ-23845 were deposited under the terms of the Budapest Treaty at the ATCC where they have been

assigned ATCC Accession Nos. PTA-1406, PTA-1407 and PTA-1408 respectively. Applicant(s) will impose no restrictions on the availability of th deposited material from the ATCC; how v r, Applicants have no authority to waiv any restrictions imposed by law on the transfer of biological material or its transportation in commerce. Applicants do not waive any infringement of its rights granted under any patents or breeder's rights granted in any country including rights in the United States under this patent and/or under the Plant Variety Protection Act (7 USC 2321 et seq.).

The foregoing invention has been described in detail by way of illustration and example for purposes of exemplification. However, it will be apparent that changes and modifications such as single gene modifications and mutations, somaclonal variants, variant individuals selected from populations of the plants of the instant lines, and the like, are considered to be within the scope of the present invention.

Claims

What is claimed is:

1. A Brassica juncea plant tolerant to a level of herbicide that prevents or inhibits the growth of a wild-type Brassica juncea plant.

2. The plant of Claim 1 wherein the herbicide is an imidazolinone herbicide.

3. The plant of Claim 1 wherein the herbicide is a sulfonyl urea herbicide.

4. The plant of Claim 1 wherein the herbicide tolerance is developed by non-transgenic means.

5. The plant of Claim 4 wherein the non-transgenic means is mutagenesis.

6. Pollen of the plant of Claim 1.

7. An ovule of the plant of Claim 1.

8. A tissue culture of the plant of Claim 1.

9. A progeny plant derived from the plant of Claim 1.

10. A progeny plant derived from the plant of Claim 1, wherein the progeny plant retains substantially all of the herbicide tolerance of the plant of Claim 1.

11. A progeny plant derived from the plant of Claim 1, wherein the progeny plant contains one or more transgenes.

12. A Brassica juncea seed that, when planted, will produce a Brassica juncea plant tolerant to a level of herbicide that prevents or inhibits the growth of a wild-type Brassica juncea plant.

13. The seed of claim 12 wherein the herbicide is an imidazolinone herbicide.

14. The seed of Claim 12 wherein the herbicide is a sulfonyl urea herbicide.

15. The seed of Claim 12 wherein the herbicide tolerance is developed by non-transgenic means.

16. A Brassica juncea plant material tolerant to a level of herbicide which prevents or inhibits the growth of wild-type Brassica juncea plant material.

17. The plant material of Claim 16 wherein the herbicide is an imidazolinone herbicide.

18. The plant material of Claim 16 wherein the herbicide is a sulfonyl urea herbicide.

19. The plant material of Claim 16 wherein the herbicide tolerance is developed by non-transgenic means.

20. The plant material of Claim 16 wherein the plant material is a full grown plant or its parts.

21. The plant material of Claim 16 wherein the plant material is an immature plant or its parts.

22. The plant material of Claim 16 wherein the plant material is a seed or its parts.

23. The plant material of Claim 22 wherein the seed or its parts are mature.

24. The plant material of Claim 22 wherein the seed or its parts are immature.

25. An herbicide tolerant Brassica juncea line designated 98SJ-23841, representative seed of the line having been deposited under ATCC accession No. PTA-1406.

26. A Brassica juncea plant or its parts produced by the seed of Claim 25.

27. Pollen of the plant of Claim 26.

28. An ovule of the plant of Claim 26.

29. A tissue culture of the plant of Claim 26.

30. A method for producing a Brassica line 98SJ-23841-derived Brassica plant, comprising:

(a) crossing Brassica line 98SJ-23841 with a second Brassica plant to yield progeny Brassica seed; and

(b) growing said progeny Brassica seed to yield the Brassica line 98SJ-23841-derived Brassica plant.

31. A Brassica plant, or parts thereof, produced by the method of Claim 30.

32. The method of Claim 30, further comprising:

(c) crossing the Brassica line 98SJ-23841-derived Brassica plant of (b) or (d) with itself or another Brassica plant to yield additional Brassica line 98SJ-23841-derived progeny Brassica seed;

(d) growing the progeny Brassica seed of step (c) to yield an additional Brassica line 98SJ-23841-derived Brassica plant; and

(e) repeating the crossing and growing steps of (c) and (d) from 0 to 5 times to produce further Brassica line 98SJ-23841-derived Brassica plants.

33. A Brassica plant, or parts thereof, produced by the method of Claim 32.

34. The plant or plant parts of Claim 33, wherein the plant or plant parts retain substantially all of the herbicide tolerance of Brassica line 98SJ-23841.

35. The plant or plant parts of Claim 34, wherein the herbicide tolerance retained by the plant or plant parts is imidazolinone tolerance.

36. The plant or plant parts of Claim 33, wherein the plant or plant parts contain one or more transgenes.

37. An herbicide tolerant Brassica juncea line designated 98SJ-23844, representative seed of the line having been deposited under ATCC accession No. PTA-1407.

38. A Brassica juncea plant or its parts produced by the seed of Claim 37.

39. Pollen of the plant of Claim 38.

40. An ovule of the plant of Claim 38.

41. A tissue culture of the plant of Claim 38.

42. A method for producing a Brassica line 98SJ-23844-derived Brassica plant, comprising:

(a) crossing Brassica line 98SJ-23844 with a second Brassica plant to yield progeny Brassica seed; and

(b) growing said progeny Brassica seed to yield the Brassica line 98SJ-23844 -derived Brassica plant.

43. A Brassica plant, or parts thereof, produced by the method of Claim 42.

44. The method of Claim 42, further comprising:

(c) crossing the Brassica line 98SJ-23844-derived Brassica plant of (b) or (d) with itself or another Brassica plant to yield additional Brassica line 98SJ-23844-derived progeny Brassica seed;

(d) growing the progeny Brassica seed of step (c) to yield an additional Brassica line 98SJ-23844-derived Brassica plant; and

(e) repeating the crossing and growing steps of (c) and (d) from 0 to 5 times to produce further Brassica line 98SJ-23844-derived Brassica plants.

45. A Brassica plant, or parts thereof, produced by the method of Claim 44.

46. The plant or plant parts of Claim 45, wherein the plant or plant parts retain substantially all of the herbicide tolerance of Brassica line 98SJ-23844.

47. The plant or plant parts of Claim 46, wherein the herbicide tolerance retained by the plant or plant parts is imidazolinone tolerance.

48. The plant or plant parts of Claim 45, wherein the plant or plant parts contain one or more transgenes.

49. A herbicide tolerant Brassica juncea line designated 98SJ-23845, representative seed of the line having been deposited under ATCC accession No. PTA-1408.

50. A Brassica juncea plant or its parts produced by the seed of Claim 49.

51. Pollen of the plant of Claim 50.

52. An ovule of the plant of Claim 50.

53. A tissue culture of the plant of Claim 50.

54. A method for producing a Brassica line 98SJ-23845-derived Brassica plant, comprising:

(a) crossing Brassica line 98SJ-23845 with a second Brassica plant to yield progeny Brassica seed; and

(b) growing said progeny Brassica seed to yield the Brassica line 98SJ-23845 -derived Brassica plant.

55. A Brassica plant, or parts thereof, produced by the method of Claim 54.

56. The method of Claim 54, further comprising:

(c) crossing the Brassica line 98SJ-23845-derived Brassica plant of (b) or (d) with itself or another Brassica plant to yield additional Brassica line 98SJ-23845-derived progeny Brassica seed;

(d) growing the progeny Brassica seed of step (c) to yield an additional Brassica line 98SJ-23845-derived Brassica plants; and

(e) repeating the crossing and growing steps of (c) and (d) from 0 to 5 times to produce further Brassica line 98SJ-23845-derived Brassica plants.

57. A Brassica plant, or parts thereof, produced by the method of Claim 56.

58. The plant or plant parts of Claim 57, where the plant or plant parts retain substantially all of the herbicide tolerance of Brassica line 98SJ-23845.

59. The plant or plant parts of Claim 58, wherein the herbicide tolerance retained by the plant or the plant parts is imidazolinone tolerance.

60. The plant or plant parts of Claim 57, wherein the plant or plant parts contain one or more transgen s.

61. A method of producing herbicide tolerance in Brassica juncea which comprises:

(a) hybridizing an herbicide tolerant Brassica napus plant and a Brassica juncea plant to produce hybrid plant material, and

(b) selecting hybrid plant material that retains the morphological or genotypic characteristics of Brassica juncea and is tolerant to a level of herbicide which prevents or inhibits the growth of a wild-type Brassica juncea plant.

62. The method of claim 61 wherein the herbicide is an imidazolinone herbicide.

63. The method of claim 61 wherein the herbicide is a sulfonyl urea herbicide.

64. The method of claim 61 wherein the Brassica juncea is the female in the hybridization.

65. The method of claim 61 wherein the Brassica juncea is the male in the hybridization.

66. The method of claim 61 wherein the herbicide tolerance of the Brassica napus plant is not developed through transgenic means.

67. The method of claim 61 wherein the hybrid plant material is a full grown plant or its parts.

68. The method of claim 61 wherein the hybrid plant material is an immature plant or its parts.

69. The method of claim 61 wherein the hybrid plant material is a seed or its parts.

70. The method of claim 61 wherein the hybrid plant material is an immature seed or its parts.

71. A method for controlling weeds growing with Brassica juncea which comprises:

(a) growing the plants of claim 2, and

(b) using an imidazolinone herbicide to control weeds.

72. A method for controlling weeds growing with Brassica juncea which comprises:

(a) growing the plants of claim 3, and

(b) using a sulfonyl urea herbicide to control weeds.

73. A Brassica juncea plant tolerant to a level of herbicide that prevents or inhibits the growth of a wild-type Brassica juncea plant, the herbicide tolerant Brassica juncea plant developed by crossing a herbicide tolerant Brassica napus plant with a Brassica juncea plant.

74. The plant of Claim 73 wherein the herbicide is an imidazolinone herbicide.

75. The plant of Claim 73 wherein the herbicide is a sulfonyl urea herbicide.

76. The plant of claim 73, wherein the herbicide tolerant Brassica napus plant was developed by non-transgenic means.

77. The plant of claim 76 wherein the non-transgenic means is mutagenesis.

78. A method of transferring a mutagenic trait into Brassica juncea, which comprises:

(a) developing a mutagenic trait in a plant of a first species other than Brassica juncea;

(b) crossing the plant of the first species containing the mutagenic trait with a Brassica juncea plant to yield Brassica juncea derived seed containing the mutagenic trait;

(c) growing the Brassica juncea d rived seed of Step (b) to yield Brassica juncea derived plants; and

(d) backcrossing the Brassica juncea derived plants with a Brassica juncea plant and repeating the backcross from 0 to 7 times to generate stable progeny plants with a Brassica juncea phenotype and the mutagenic trait of the plant of the first species.

79. The plant produced by the method of Claim 78.

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(19)(CA) **CANADIAN PATENT** (12)

(54) Microspore-Based Selection System and Products Thereof

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(30) (US) U.S.A. 030,987 1987/03/27

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NO DRAWING

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MICROSPORE-BASED SELECTION SYSTEM AND PRODUCTS THEREOF

Background of the Invention

The present invention relates to a process for *in vitro* exploitation of genetic variability in segregating gametic tissue, as well as variability induced by the application of mutagenic agents to gametic tissue, for the purpose of selecting mutant phenotypes. The present invention also relates to a selection procedure utilizing plant cells, including protoplasts, that are developed from microspores or from microspore-derived embryos.

The isolation of novel plant mutants by the application of selective growth conditions to cultured cells is well known. For example, Chaleff and Ray, "Herbicide-Resistant Mutants from Tobacco Cell Cultures," *Science* 223: 112-15 (1984), report isolating from cultured tobacco (*Nicotiana tabacum*) cells several mutants to the herbicides chlorsulfuron (Glean®) and sulfometuron methyl (Oust®), both products of E.I. du Pont de Nemours & Co.

(Wilmington, Delaware, U.S.A.). The preparation of herbicide-tolerant tobacco, rice, corn, potato, oats, alfalfa, carrot and sugar cane plants is also the subject of U.S. patent No. 4,443,971. The patent states, in particular, that a plant from among the foregoing types which is relatively tolerant of a herbicide selected from picloram, paraquat, 2,4-D, glyphosate, alachlor, atrazine and amitrole can be obtained by culturing tissue of a herbicide-sensitive parent plant in the presence of enough herbicides to kill at least 90% of the tissue initially present.

In these known selection systems, "tissue culturing" entails propagation of plant tissue, cells or protoplasts in vitro, under selective growth conditions, and eventual regeneration of a whole plant therefrom. Crucial to the second step is the production of callus, a soft parenchymatous tissue comprised of large, thin-walled, rapidly dividing cells that can undergo differentiation to form other, more specialized tissues. In accordance with conventional selection systems, shoot formation from callus is induced, typically upon transferring callus tissue to a medium that contains an auxin like indole-3-acetic acid (IAA), a cytokinin or some other plant hormone. The shoots can then be grown into mature, whole plants which may express a trait, such as herbicide resistance, selected for at the cellular level. In this fashion, variability in the response of cultured tissue, cells or protoplasts to selective growth conditions is manifested in whole plants,

which then are used as a source for the selected trait(s) in a breeding program.

The variation upon which the known selection systems draw is essentially somaclonal in nature, i.e., the variability is the result of spontaneous genetic changes that occur in somatic cells grown in tissue culture. The possible mechanisms which may underlie somaclonal variation -- changes at the individual-nucleotides level in a DNA molecule; the movement of transposable elements within chromosomes; more massive chromosomal abnormalities, including the loss or duplication of chromosome sections and the trading of segments between chromosomes -- have yet to be elucidated in detail. Nevertheless, it is known that a large, even predominant fraction of somaclonal variation arises during the period of tissue culture, and therefore does not result from some "unmasking" of variation present in the parent plant. Where mutagens are applied to these systems, the resulting variation is a mixture of somaclonal and mutagen-induced variability.

Tissue culture itself thus represents a severe perturbation to normal development and, hence, is responsible for much of the variability that is exploited by conventional in vitro selection systems. This presents a disadvantage because that variability is difficult, if not impossible, to control. Thus, the products of somaclonal variation are characteristically very poorly defined genetically. In particular, they frequently involve at least one decidedly undesirable trait, such as aneuploidy, polyploidy, genetic rearrangement (inversions,

translocations) and other lethal or sub-lethal deficiencies, which detract from the utility of the plants regenerated therefrom.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a selection system for generating plant variants that does not rely primarily on somaclonal variation or where somaclonal variation may be one source of variation, the cells or protoplasts are derived directly from the gametic cell or resultant embryo.

It is also an object of the present invention to provide a process for exploiting genetic variation, in vitro, which process utilizes gametic cells, or tissue derived from embryos developed from gametic cells, as a tissue source.

It is another object of the present invention to provide a readily accessible source of desirable traits for rapid incorporation into important agronomic plants, such as rapeseed and other cruciferous crops.

It is a further object of the present invention to provide seed from which can be grown a plant that exhibits a desirable trait, such as tolerance to a herbicide.

In accomplishing the foregoing objects, there has been provided, in accordance with one aspect of the present invention, a process for producing plant variants, comprising the steps of (A) obtaining microspores in culture from a parent plant,

(B) generating plant embryos from microspores in the culture and (C) using the plant embryos to produce a whole plant, wherein the microspores, the plant embryos, or cells derived from the embryos are exposed to a selection agent that has a selective effect on the viability of microspores or plant cells, such that certain but not all of the exposed microspores, embryos or cells are viable. In one preferred embodiment, the whole plant generated from the plant embryos is, unlike the parent plant, tolerant to the selection agent used. In another preferred embodiment, cells derived from the embryos in the form of protoplasts are exposed to the viability-affecting selection agent.

In accordance with another aspect of the present invention, plants have been provided that are the product of the above-described process, for example, where the agent is a sulfonylurea or an imidazolinone herbicide and the plant displays a tolerance of at least one of a sulfonylurea and an imidazolinone herbicide, which tolerance is not naturally-occurring. A rapeseed plant has been provided, for example, that displays a tolerance to chlorsulfuron, a sulfonylurea herbicide, that does not occur naturally in rapeseed.

Also provided, inter alia, is a plant, as well as seeds thereof, that displays a tolerance to an imidiazolinone herbicide, which tolerance is independent of the presence in the plant of acetolactate synthase (ALS) enzyme that is tolerant of the herbicide. In a preferred embodiment, the plant does not display a tolerance of herbicides,

other than the aforesaid imidiazolinone, that bind ALS enzyme.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Detailed Description of the Preferred Embodiments

The present invention involves the efficient production of a large number of microspores, the development therefrom of a large number of haploid plant embryos, and the direct regeneration from those embryos of whole plants which express a desired trait. The term "microspore" is used here to denote the smaller spore of a heterosporous plant (that is, a plant having both a megaspore and a microspore) which gives rise to a male gametophyte, the normal developmental product of which is the pollen grain. In accordance with the present invention, at least one selection agent, preferably in conjunction with a mutagenic agent, is employed to produce a subpopulation of microspores (or other plant cells obtained using tissue developed from microspores) that is characterized by the trait(s) desired for the regenerated plants.

It has been discovered that the microspore-based selection system of the present invention is sensitive to a broad range of chemicals, which can be used for selection in the system, with or without mutagenesis, at virtually any level of development of the embryos or plants. Perhaps more importantly, it has been found that the microspores used in the selection system of the present invention, since they represent the starting point in a direct path to whole plants via embryogenesis rather than somatic cloning, provide a tissue source that is more highly correlated (in terms of characterizing properties) with tissue in the whole-plant product than are tissue sources in known selection system. Undifferentiated growth, a major disadvantage in conventional in vitro systems, is thus all but eliminated in the present invention. Moreover, the frequency with which desirable mutants are obtainable pursuant to the present invention -- typically, at a rate of 1 in about 10^4 to 10^5 microspore-derived embryos -- is readily accommodated in the context of in vitro microspore culture.

For purposes of this description, the phrase "selection agent" is used to designate a growth factor, either chemical or physical, that selectively influences the viability of cultured microspores to the effect that a subpopulation of those microspores is favored, after exposure to the agent, in subsequent embryogenesis. The phrase "mutagenic agent" refers to a growth factor that increases the level of variation in the properties of cultured microspores (or other plant cells in the system) which are

exposed to the agent, but that does not work an unduly adverse effect on embryo development.

Selection agents suitable for use in the present invention include cytotoxic chemicals such as the sulfonylurea herbicides, e.g., chlorsulfuron and sulfometuron methyl, and imidazolinone herbicides like AC 263,499 (Pursuit®; product of American Cyanamid). For purposes of the present invention, the sulfonylurea and imidazolinone herbicides are grouped together, despite their different chemistry, because both types of herbicides disrupt plant metabolism by acting on acetolactate synthase (ALS), also known as acetohydroxyl acid synthase (AHAS), the first enzyme in the pathway leading to production of leucine, isoleucine and valine in vivo.

Brassica plants are generally not known to display a natural tolerance to ALS-impairing herbicides, with the possible exception of a naturally-occurring resistance to the compound DPXY7881 (product of E. I. du Pont Co.). In the present description, the term "resistance" is used to denote the normal development of a plant after a usually effective dosage of a given selection agent has been applied. "Tolerance" pertains to a level of survival that is above a species norm but below complete resistance. Resistance to a given herbicide or other selection agent is thus the maximum tolerance at a particular level of the agent.

Exemplary of the other cytotoxic chemicals which can be used according to the present invention are paraquat, basagran, picloram, acifluorfen, 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate,

alachlor, atrazine, cycloate, Basta (a product of Hoechst Co.), glufosinate-ammonium and amitrole.

As described in greater detail below, chemical hybridizing agents, such as those agents that have gametocidal activity ("gametocides"), like RH-531, RH-532 and RH-2956 (all products of Rohm-Haas Co., Nutley, New Jersey, U.S.A.), (2-chloroethyl)phosphonic acid (Ethrel) and cupferron, are suitable selection agents in the present invention. Similarly suitable are other substances that alter gametic development, such as the halogenated aliphatic acids α,β-dichloro-isobutyrate (FW-450; product of Rohm-Haas Co.) and sodium 2,2-dichloropropionate (Dalapon®), antiauxins like maleic hydrazide and triiodobenzic acid, auxins like naphthaleneacetic acid and (at non-cytotoxic levels) 2,4-D, and gibberellins like GA4.

In another preferred embodiment, a host-specific toxin (HST) from a plant pathogen is employed in the present invention as a selection agent. Particularly preferred in this regard are the HSTs produced by saprophytic pathogens like Helminthosporium and Alternaria (see Table 1 below), which compounds can be used in the present invention to develop pathogen-resistant variants of tomato, citrus and other agronomic crops. In yet another preferred embodiment, the selection agent takes the form of an environmental factor, such as high and low temperatures, culture-medium salinity or pH, which can influence in vitro development and for which tolerance is desired.